N-acetylcysteine protects against bupivacaine-induced myotoxicity caused by oxidative and sarcoplasmic reticulum stress in human skeletal myotubes.

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Financial support

INSERM ERI 25, F-34295 Montpellier, France, Université Montpellier 1, Montpellier, France, La Région Languedoc-Roussillon, Montpellier, France, L'Association Française contre les Myopathies, Paris, France

Acknowledgements

We would like to thank the Tissue Bank for Research (MYOBANK-AFM, Paris, France) of the Association Française contre les Myopathies (AFM) for generously providing human muscle biopsies.

Abbreviated Title

NAC protects against bupivacaine-induced apoptosis

Brief Summary

In human skeletal muscle myotubes, bupivacaine-induced myotoxicity is associated with reactive oxygen species production, oxidative stress and sarco/endoplasmic reticulum stress. N-acetyl cysteine protects against these iatrogenic effects in human myotubes.

Abstract

Background: Local anesthetics offer the benefits of extended analgesia with greater patient satisfaction and faster rehabilitation compared with intravenous morphine. These benefits, however, can be offset by adverse iatrogenic muscle pain. Here, the authors investigate the mechanisms of local anesthetic-induced myotoxicity and assess the protective effect of N-acetyl cysteine.

Methods: The authors used primary cell cultures of human skeletal muscle myoblasts to study local anesthetics adverse effects. Production of reactive oxygen species was investigated in human skeletal myotubes by fluorescence microscopy. Expression of sarcoplasmic/endoplasmic reticulum stress markers and induction of apoptosis were followed by immunofluorescence and western blot analysis. Finally, the effect of N-acetyl cysteine on bupivacaine-induced myotoxicity was investigated in vitro.

Results: Bupivacaine sequentially induced reactive oxygen species production, oxidative stress, sarcoplasmic/endoplasmic reticulum stress and activation of Caspases-9 and -7 in human differentiated myoblasts. These iatrogenic effects were prevented by N-acetyl cysteine.

Conclusions: The authors demonstrated a protective effect of N-acetyl cysteine against bupivacaine-induced sarcoplasmic/endoplasmic reticulum stress and apoptosis in primary human skeletal muscle cell.

Introduction

Local anesthetics (LAs) offer the benefits of extended analgesia with greater patient satisfaction compared with intravenous morphine after orthopedic surgery.[1-3] Challenges remain, however, for the use of LAs to improve the comfort and postoperative pain relief in patients who receive continuous regional blocks for surgical procedures. These benefits, however, can be offset by adverse iatrogenic muscle pain caused by bupivacaine.[4,5] Local anesthetic-induced myotoxicity seems to be a rather uncommon side effect of local and regional anesthesia. However, studies revealed that certain anesthesia techniques, especially retrobulbar and peribulbar blocks, are related to a relatively high postoperative rate of significant muscular dysfunction directly caused by these agents.[6] Therefore, skeletal muscle damage has to be considered a potentially serious complication after local anesthetic application. The frequency of these symptoms is largely unknown because they remain underreported.[6] Moreover, a better understanding of the mechanisms of LA-induced myotoxicity is needed in order to develop efficient clinical strategies to protect against LA adverse outcomes.

After LA administration the affected muscle fibers undergo an intrinsic degenerative phase with increase in pycnotic myonuclei and pathologically condensed chromatin. However the cellular mechanisms, particularly the apoptotic pathway(s) involved in LA myotoxicity, remain to be characterized (reviewed in [7]). Several studies indicate that in lidocaine-induced neurotoxicity the intrinsic mitochondrial death pathway, rather than the extrinsic death receptor pathway, might be implicated in the apoptotic response to LA administration.[8,9] In addition, bupivacaine could interfere with the mitochondrial energy metabolism in skeletal muscle,[10,11] leading to Ca^{2+} deregulation. It has been proposed that increased intracellular Ca^{2+} concentrations play a role in myocyte injury.[12,13] Indeed, in skinned fiber preparations of skeletal muscle, LAs cause Ca^{2+} release from the sarcoplasmic reticulum (SR) and simultaneously inhibit Ca^{2+} reuptake,[14-16] thus resulting in persistently increased Ca^{2+} levels. On the other hand, in psoas muscle of rats anaesthetized with bupivacaine and ropivacaine

administered by catheter for femoral nerve block, a decrease in the mitochondrial energy metabolism was detected, but without changes in SR Ca^{2+} content.[5] Interestingly, a microarray analysis revealed that, in promyelocytic leukemia cells HL60, bupivacaine treatment upregulated the expression of several apoptosis-related genes and also of X-box Binding Protein 1 (XBP-1).[17] XBP-1 is a transcription factor that operates as a downstream component of the unfolded protein response against sarco- or endo-plasmic reticulum (SR/ER) stress due to accumulation of unfolded/misfolded proteins.[18] XBP-1 also controls expression of C/EBP Homologous Protein (CHOP),[19] a key-signaling component of ER stress-induced apoptosis, which, therefore, could mediate bupivacaine-induced myotoxicity.

To better characterize the mechanism(s) of LA-induced myotoxicity and, particularly, the involvement of SR/ER stress, we investigated bupivacaine effects in human primary myotubes. We have identified the Caspases activated during the apoptotic response to bupivacaine and demonstrated that bupivacaine-induced apoptosis is mediated by SR stress due to increased reactive oxygen species (ROS) production. Finally, we report that N-acetyl cysteine (NAC) protects against these iatrogenic effects.

Materials and Methods

Reagents

Anti-Tubulin monoclonal antibody (mAb) was from Sigma Aldrich® (Sigma Aldrich®, St. Quentin Fallavier, France); anti-CHOP, -XBP-1 and -ATF-6 (activating transcription factor 6) polyclonal antibody (pAb) were from Abcam® (Abcam®, Paris, France); anti-CHOP mAb, anti-PARP (Poly (ADP-ribose) Polymerase) and anti cleaved-PARP, anti-Caspases 9,7,6,3 and anti-cleaved Caspases 9, 7, 6, 3 pAb were from Cell Signaling Technology® (Ozyme, Montigny, France). The Alexa Fluor® secondary antibodies were purchased from Invitrogen™ (Invitrogen™, Cergy Pontoise, France). Bupivacaine (1-Butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide), staurosporine, thapsigargin and N-acetyl cysteine were purchased from Sigma Aldrich and CM-H2DCFDA derivatives (5- (and6)-chloromethyl-2',7' -dichlorodihydrofluorescein diacetate, acetyl ester) from Invitrogen™.

Primary cell cultures

Human primary myoblasts were isolated as described previously [20] from quadriceps muscle biopsies obtained from the ‘‘AFM-BTR Banque de Tissus pour la Recherche’’ (Hôpital de la Pitié-Salpêtrière, 75013 Paris). 30 000 myoblasts/ml were grown in growth medium (Dulbecco's Modified Eagle Medium (Cambrex, Lonza, Verviers, Belgium) supplemented with 10% foetal calf serum (Perbio Science, Brebieres, France) and 1% Ultroser® G (PALL Life Sciences, Cergy St. Christophe, France) for five days. At day 5, myoblasts started to fuse and cultures were switched to differentiation medium composed of Dulbecco's modified eagle medium supplemented with 10% foetal calf serum and Penicillin/Streptomycin (Cambrex, Lonza, Verviers, Belgium). Cultures of myotubes grown in differentiation medium were used for all the experiments.

Immunoblotting and Immunofluorescence analysis

Cell extracts prepared as described previously [21] were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes and probed with the indicated antibodies. Bound antibodies were detected with a chemiluminescence detection kit (PerkinElmer®, Courtaboeuf, France). Analysis and quantification were performed with Kodak 1D3.6 software (Kodak, Carestream Health France, Bagnolet, France) .

For immunofluorescence, cells were fixed in 4% paraformaldehyde in phosphate buffered saline and permeabilized with 0.1% Triton X-100 in phosphate buffered saline. Cells were incubated with primary antibodies for 2h, followed by Alexa Fluor® secondary antibodies and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cell imaging was performed at the "Centre Regional d'Imagerie Cellulaire de Montpellier". Fluorescent cells were viewed with a Leica Microscope (Leica DM6000, Wetzlar, Germany) using a 40x/ NA=1.30 EC Plan-Neofluar grade oil objective. Images were captured as 16 TIFF files with a MicroMax 1300 CCD cameras (RS-Princeton Instruments Inc., Roper Scientific SAS, Evry, France) driven by the MetaMorph® (version 7; Universal Imaging Corp., Roper Scientific SAS, Evry, France). Images were processed using ImageJ software (National Institutes of Health, Bethesda, MD; ImageJ is in the public domain).

ROS production and oxidative stress analysis

We used CM-H2DCFDA (5- (and6)-chloromethyl-2',7' -dichlorodihydrofluorescein diacetate, acetyl ester) as cell-permeant indicator of ROS. Myotubes were cultured in 6-well plates, treated with 0.5, 1.0 and 1.5 mM bupivacaine for 8 hours, washed and incubated with 5 µM CM-H2DCFDA (5-(and6)-chloromethyl-2',7' -dichlorodihydrofluorescein diacetate, acetyl ester) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in phosphate buffered saline at 37°C in the dark for 60 min. Cells were then washed with phosphate buffered saline three times, fixed in 4% paraformaldehyde for 5 min and stored at 4°C for up to 16 h before image acquisition. Cells were visualized with Zeiss (Carl Zeiss SAS, Le Pecq, France) fluorescent microscope (AxioCam MRm equipped with a charged-coupled device camera), using excitation sources and filters appropriate for fluorescein (FITC) as

described by the manufacturer. Fluorescence quantification was performed with Image J using for each experimental condition ten images from at least three independent experiments (30 images, > 300 myotubes analyzed in total/experimental condition), were then combined to allow quantitative analysis of changes in ROS levels. In all experiments, fluorescence levels were normalized by the number of nuclei.

The OxyBlot™ Protein Oxidation Detection Kit was purchased from Millipore™ (Division Bioscience Millipore SAS, St Quentin en Yvelines, France) and analysis of the oxidation status of protein samples (dinitrophenylhydrazine (DNPH) derivatization) was performed following the manufacturer's instructions. One dimensional electrophoresis was carried out using 12.5% sodium dodecyl sulfate polyacrylamide gels after dinitrophenylhydrazine (DNPH) derivatization. Proteins were transferred onto nitrocellulose membranes and then stained with Red Ponceau to control for sample loading. After labeling with the rabbit anti-DNP antibody included in the kit, blots were developed using a chemiluminescence detection system.

Statistical analysis

Distribution of the quantitative variables was fitted. Transformation to ensure normal distribution was performed when necessary. Means were compared between each experimental conditions by applying a one-way analysis of variance (ANOVA) or a unpaired *t*-test when *a priori* comparisons were performed *versus* control. When ANOVA was significant, *post-hoc* unpaired *t*-test were applied and *P* adjusted to control the false discovery rate according to the Holm's procedure. All *P* values were two tailed and were considered significant at 0.05 or less. All data are presented as mean ± SEM and the statistical analysis was performed using Stata Statistical Software v6.0 (Stata Corporation, College Station, TX).

Results

Bupivacaine induces apoptosis of human skeletal muscle cells and the cleavage of Caspases 9 and 7

The myotoxicity of the LA bupivacaine was investigated in primary cell cultures of human myoblasts that fused in vitro to form mature differentiated myotubes. As the cleavage of Poly (ADP-ribose) Polymerase (PARP) is an indicator of apoptosis, the presence of its cleaved form was determined by immunofluorescence in myotubes incubated with different concentrations (from 0.75 to 1.5 mM) of bupivacaine and with 1 µM of stauropsorine, a known initiator of apoptosis in vitro, for 18 h. Cleaved PARP was clearly detected in myotubes treated with staurosporine and in cells incubated with 1.25 and 1.5 mM bupivacaine (Fig. 1A). In addition, the number of PARP-positive nuclei in myotubes treated with 1.25 mM bupivacaine increased proportionally to the duration of the incubation (Fig. 1B). Thus, induction of apoptosis by bupivacaine is dose- and time-dependent in primary human myotubes. We then assessed whether and which Caspases were implicated in bupivacaine-induced apoptosis by analyzing the expression of their cleaved forms. Immunoblot analysis showed that cleaved PARP and cleaved Caspase 9 and 7 were detected in human myotubes treated with 1.5-2 mM bupivacaine but not in cells treated with 1mM bupivacaine (Fig. 2A) consistently with the results described in Figure 1. Similarly, expression of cleaved Caspase 9 and 7 increased with longer incubation times (Fig. 2B and 2C). Cleavage of Caspase 3 and 6 was not induced by bupivacaine differently from staurosporine which induced strong expression of all the cleaved Caspases tested (Fig. 2A). These results indicate that bupivacaine induces apoptosis in human myotubes by a mechanism probably different from that of staurosporine.

Bupivacaine induces activation of the unfolded protein response and CHOP expression.

As the activation of Caspase 7 is involved in endoplasmic reticulum stress-mediated apoptosis,[22] we next tested whether bupivacaine myotoxicity could be mediated via induction of SR/ER stress in

human myotubes. To this aim, myotubes were incubated with different concentration (from 0.75 to 1.5 mM) of bupivacaine for 18 hours and with 100 nM of thapsigargin (T), an irreversible inhibitor of the endoplasmic reticulum Ca2+-ATPase, for 4 hours. Expression of CHOP,[23-25] a key downstream transcription factor in SR stress-induced apoptosis, was stimulated by thapsigargin (Fig. 3A). Bupivacaine (1-1.5mM) also strongly upregulated CHOP expression (Fig. 3A) and this effect was dose-dependent (Fig. 3B). Moreover, 1mM bupivacaine induced CHOP expression (Figure 3A, 4A and 4B) but not activation of Caspase 9 and 7 (Fig. 4A and 4 B) as observed also with thapsigargin. Thus, induction of SR stress seems to precede activation of Caspases. To test this hypothesis, the kinetics of the expression of XBP-1 and ATF-6 (two transcription factors that control expression of the unfolded protein response genes like CHOP), CHOP and cleaved Caspase 9 and 7 were determined in myotubes treated with 1mM bupivacaine. ATF-6, XBP-1 and CHOP were detected after 8 hours of bupivacaine treatment and their expression was maintained, except for XBP-1 which was transient (Figure 4C and 4D). Cleaved Caspases were detected only at 24 hours (Fig. 4C and 4D). Together, these results show that following exposure to bupivacaine, SR stress and expression of the unfolded protein response genes are induced before apoptosis, suggesting that the apoptotic events depend on SR stress induction.

Production of ROS and oxidative stress are increased in human myotubes treated with bupivacaine.

Reactive oxygen species (ROS) alter cellular redox reactions, interfere with protein disulphide bonding and result in protein misfolding leading to SR stress and the unfolded protein response activation (reviewed in [26]). Therefore, primary myotubes were incubated with different concentration (from 0.5 to 1.5 mM) of bupivacaine for 8 hours and ROS production was evaluated indirectly by measuring the oxidation status of CM-H_2DCFDA derivatives. Dichloro-fluorescein oxidation was significantly increased in myotubes treated with 1mM and 1.5mM bupivacaine compared to control, untreated, cells (Figure 5A). Moreover, ROS production in bupivacaine-treated myotubes was

inhibited by the antioxidant drugs N-acetyl cysteine, trolox (a water-soluble derivative of vitamin E) and resveratrol (antioxidant polyphenol) in a dose-dependent manner (Figure 5B and data not shown). To confirm that bupivacaine-induced ROS caused oxidative stress in myotubes, the content in carbonylated proteins of samples treated with 1.5 mM bupivacaine for 8 hours was assessed. As shown in Figure 5C, carbonylated proteins increased in response to bupivacaine treatment and this effect was prevented by anti-oxidant treatment.

N-acetyl cysteine (NAC) protects against SR stress and apoptotic events induced by bupivacaine.

To test whether inhibition of ROS production could protect against bupivacaine-induced SR stress and apoptosis, primary myotubes were incubated with 1 or 1.5 mM of bupivacaine and increasing concentrations of the anti-oxidant NAC for 18 hours. Addition of NAC decreased CHOP expression and inhibited expression of activated Caspases in myotubes (Figure 6A and 6B). CHOP expression was also monitored by immunofluorescence and the percentage of nuclei expressing CHOP decreased from 48% in bupivacaine-treated myotubes to 26%, 16% and 9% in cells co-incubated with 2, 5 and 10 mM NAC, respectively (Figure 6C).

These results show that, in primary human myotubes, blocking ROS production with the anti-oxidant NAC has a protective effect against bupivacaine-induced myotoxicity. These findings could have an important clinical impact, which will have to be evaluated in the practice.

Discussion

A variety of experimental findings indicate that LA myotoxicity has a complex pathogenesis. The experiments described indicate that treatment of human primary myotubes with bupivacaine induces ROS production and SR stress leading to apoptosis characterized by expression of cleaved Caspase 9 and 7. Co-incubation of myotubes with bupivacaine and anti-oxidant drugs, especially NAC, inhibits ROS production and SR stress and consequently Caspase activation (Fig. 7).

Skeletal muscle fiber degeneration induced by bupivacaine has been confirmed in multiple studies but the pathway of cell death remained to be clarified,[7] In neuroblastoma cells, the LA lidocaine induces a cell death,[8,9] that is prevented by the lack of Caspase 9.[9] indicating an apoptosis mediated by the intrinsic mitochondrial death pathway. Consistent with these observations, we detected the cleaved form of PARP, which is an indicator of apoptosis, and expression of activated Caspase 9 and 7 in human myotubes treated with bupivacaine, suggesting the involvement of the intrinsic pathway. LA also can decrease the mitochondrial membrane potential,[10,11] leading to progressive fragmentation of the mitochondrial network in human myoblasts.[27] However, mitochondria are unlikely to be the only targets of LAs. The increase of the intracellular Ca^{2+} concentrations by LAs could play a role in myocyte injury by inducing pathways of cell death.[12,13] In isolated myofibers, bupivacaine induces Ca^{2+} release from the SR by acting on the Ca^{2+} release channel- ryanodine receptors at SR membranes, and inhibits Ca^{2+} reuptake into the SR, which is mainly regulated by SR Ca^{2+} ATPase activity.[16,28] Bupivacaine could inhibit these receptors by direct binding, but this has never been demonstrated. Therefore, and based on the results of this work, we propose an alternative mechanism linked to induction of oxidative stress. In human myotubes, bupivacaine treatment increases production of ROS which may then modify oxidizable residues (cysteine, tyrosine) of SR-associated calcium channels, such as ryanodine receptors (S-nitrosylation) and SR/ER Ca^{2+} ATPases (by tyrosine

nitration), causing their dysfunction and SR calcium depletion.[29-31] However, disturbance of intracellular Ca^{2+} homeostasis should not be considered the only mechanism by which bupivacaine induces apoptosis in muscle. Indeed, in vivo, bupivacaine injected via femoral nerve block catheters has deleterious effects on mitochondrial energy without affecting calcium homeostasis[5] and, in our experiments, bupivacaine induced apoptosis at concentrations that do not influence Ca^{2+} release from SR. Another mechanism, associated with SR/ER stress, should therefore be considered. Here, we show that, in myotubes, bupivacaine induces activation of Caspase 7, which is involved in ER stress-mediated apoptosis,[22] as well as expression of the ER stress markers XBP-1, ATF-6 and CHOP. Oxidative stress can cause SR/ER stress. Production of ROS, which alter cellular redox reactions, and production of nitric oxide, a mediator of protein nitrosylation, interfere with protein disulphide bonding and result in protein misfolding.[26] The excessive presence of misfolded proteins disrupts SR/ER function, resulting in accumulation of unfolded or misfolded proteins in the reticulum lumen which leads to SR/ER stress and the unfolded protein response (reviewed in [19]). In bupivacaine-treated myotubes, addition of the antioxidant N-acetyl cysteine protects against ROS production, as expected, but also inhibits expression of CHOP and of activated Caspases. These results strongly suggest that bupivacaine causes muscle injury by increasing ROS production which leads to SR/ER stress and finally to apoptosis. The implication of ROS production in bupivacaine-mediated toxicity was also shown in a schwann cell line,[32] but how bupivacaine induces ROS production remains a major question for future studies.

Furthermore, the dose of 1 to 2 mM bupivacaine used for the experiments described in this paper was chosen to mimic the effects of direct exposure of myotubes to this agent at clinically relevant concentrations (7.7 mM = 0.25% bupivacaine solution used in peripheral nerve blocks). These concentrations are in the same range than those which altered mitochondrial energy metabolism in human myoblasts (1-5 mM bupivacaine),[27] and induced apoptosis in a Schwann cell line (0.5 mM

bupivacaine).[32] In vivo, lipophilic local anesthetics accumulate in tissues but the real concentrations in the cells remain unknown. Bupivacaine concentration in the muscle 1 hour after the last injection of a clinically relevant protocol performed in rats was around 30 nmol/mg of tissue or around 30 µM.[5] Nevertheless, this concentration is underestimated because the authors could not measure precisely the local concentration of bupivacaine in the strict diffusion space within the muscle due to the limited sensitivity of the high-performance liquid chromatography system that would have required a larger biopsy. Thus, 1mM bupivacaine is probably not so far away from real concentrations in the vicinity of the nerve catheter. Anyhow, in clinical practice, the relevance of concentration depends mainly on the dose-dependant effect, which play a key-role in bupivacaine toxicity. This suggests that the current clinical protocols for local anesthetic administration should be re-evaluated and adapted in order to use dose close to the minimal local anesthetic concentration needed.

The observation that an antioxidant protects against LA-induced myotoxicity constitutes an important result for the clinical practice. Recently, we showed that recombinant human erythropoietin (rhEPO), often used for its hematopoietic effects, can partially protect against LA-induced myotoxicity, in rats and cultured human myoblasts [27]. Nevertheless, the recombinant human erythropoietin (rhEPO) doses used in these experiments were much higher than those typically used clinically to treat anemia [33]**.** Therefore, the risk of side effects combined with its extremely high cost, does not make of recombinant human erythropoietin (rhEPO) an ideal treatment against LA-induced myotoxicity. Conversely, N-acetyl cysteine might be an interesting alternative, although the clinical impact of our results remains to be evaluated in the practice.

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Figure Legends

Figure 1: Bupivacaine-induced apoptosis in human primary myotubes.

(A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine or 1µM staurosporine (S) as a positive control. After 18 hours, myotubes were fixed and incubated with anti-cleaved poly (ADP-ribose) polymerase (PARP) antibodies, followed by fluorescently-labeled secondary antibodies. DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and F-Actin with rhodamin-conjugated phalloidin. (B) After 18 hours (18h), 24 hours (24h) and 31 hours (31h), the percentage of nuclei positive for cleaved poly (ADP-ribose) polymerase (PARP) was determined. Values are the mean ± SEM (n=4); $P < 0.05$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* control (Ctl) with Holm's procedure. Scale bars: 10 µm.

Figure 2: Bupivacaine-induced Caspase activation in human primary myotubes.

(A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine or 1µM staurosporine (S) as a positive control. After 18 hours, myotubes were lysed and immunoblotted with the indicated antibodies. Analysis of Tubulin expression served as loading control. (B) Human myotubes were treated with 1.5mM bupivacaine and Caspase activation was analyzed at the indicated time points. (C) The histograms show the quantification of the expression level of the proteins obtained in experiments (A) and (B). Data are the mean ± SEM (n=4); $P < 0.05$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* without bupivacaine for the same time with Holm's procedure. Bupi = Bupivacaine, PARP = poly (ADP-ribose) polymerase.

Figure 3: Bupivacaine-induced CHOP expression in human myotubes.

(A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine and 1µM staurosporine (S) or 100 nM thapsigargin (T), a known inducer of C/EBP homologous protein (CHOP) expression and sarco/endoplasmic reticulum stress. After 18 hours, myotubes were fixed and incubated with anti-CHOP antibody as indicated, followed by fluorescently-labeled secondary antibodies. DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). (B) The percentage of CHOP-positive nuclei was determined in fluorescent experiments. Values are the mean ± SEM (n=4); $P < 0.05$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* control (Ctl) with Holm's procedure; $^{\S}P < 0.05$ with unpaired *t*-test *versus* control (Ctl). Scale bars: 10 µm.

Figure 4: Bupivacaine-induced CHOP expression precedes Caspase activation.

(A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine, 1µM staurosporine (S) or 100 nM thapsigargin (T). After 18 hours, myotubes were lysed and immunoblotted using the indicated antibodies. (B) Histograms show the quantification of the experiments described in (A). Data are the mean ± SEM (n=4); $P < 0.05$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* without bupivacaine with Holm's procedure. (C) Myotubes treated with 1mM bupivacaine were lysed at different time points as indicated. Cell extracts were immunoblotted using the indicated antibodies. Analysis of Tubulin expression served as loading control. The active and inactive forms of ATF-6 are indicated and * marks non-specific bands. (D) Histograms show the quantification of the experiments described in (C). Data are the mean ± SEM (n=4); $P < 0.05$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* without bupivacaine for the same time with Holm's procedure. cl-CSP9 = Cleaved Caspase-9, cl-CSP7 = Cleaved Caspase-7. CHOP = C/EBP homologous protein, PARP = poly (ADP-ribose) polymerase, ATF-6 = activating transcription factor 6, XBP-1 = X-box binding protein 1.

Figure 5: Antioxidants protect against bupivacaine-induced oxidative stress.

(A) Reactive oxygen species (ROS) production was assessed by oxidation of CM-H2DCFDA (5-(and6)-chloromethyl-2',7' -dichlorodihydrofluorescein diacetate, acetyl ester) derivatives in myotubes treated, or not (Ctl), with the indicated concentrations of bupivacaine for 8 hours. Values are the mean ± SEM (n=4); $P < 0.01$ with one-way analysis of variance and $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* control (Ctl) with Holm's procedure. (B) Reactive oxygen species (ROS) production was measured in myotubes incubated, with the indicated concentrations of bupivacaine, N-acetyl cysteine (NAC) for 8 hours. Values are the mean ± SEM (n=4), NAC effect is analysed for the indicated concentrations of bupivacaine, $P < 0.01$ with one-way analysis of variance; $*P < 0.05$ *with post hoc* unpaired *t*-test *versus* control (Ctl) with Holm's procedure; $^{\#}P < 0.05$ *with post hoc* unpaired *t*-test *versus* without N-acetyl cysteine (baseline value) for the same bupivacaine concentration, with Holm's procedure. (C) Oxyblot analysis of carbonylated proteins from myotubes treated with vehicle (Ctl, lane1) or with 1.5mM of bupivacaine (lanes 2, 3 and 4) and the indicated concentrations of NAC (lanes 3 and 4) for 8 hours. Histograms show the quantification of carbonylated proteins. Data are the mean ± SEM (n=4); $*P < 0.05$ *with* unpaired *t*-test v*ersus* control (Ctl, line 1); $P < 0.01$ with one-way analysis of variance and $^{\#}P < 0.05$ *with post hoc* unpaired *t*-test *versus* without N-acetyl cysteine (baseline value) for the same bupivacaine concentration, with Holm's procedure.

Figure 6: NAC protects against the iatrogenic effects of bupivacaine in human myotubes.

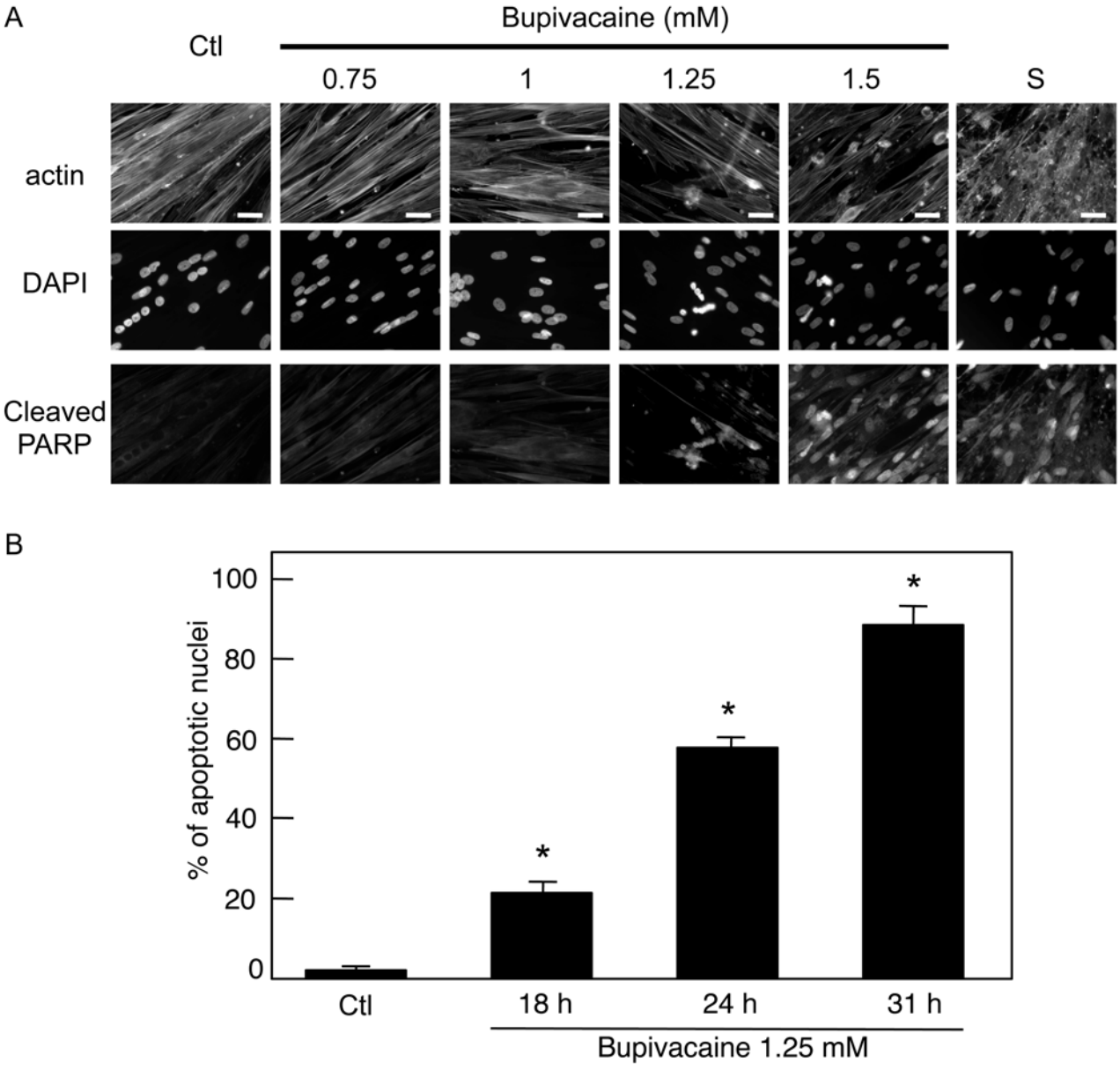
(A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine, 1µM staurosporine (S), 100 nM thapsigargin (T) and N-acetyl cysteine (NAC). After 18 hours, myotubes were lysed and immunoblotted using the indicated antibodies. (B) Histograms show the quantification of the experiments described in (A). Data are the mean ± SEM (n=4), for CHOP expression analysis; for 1 and 1.5 mM bupivacaine, $*P < 0.01$ with unpaired *t*-test versus control

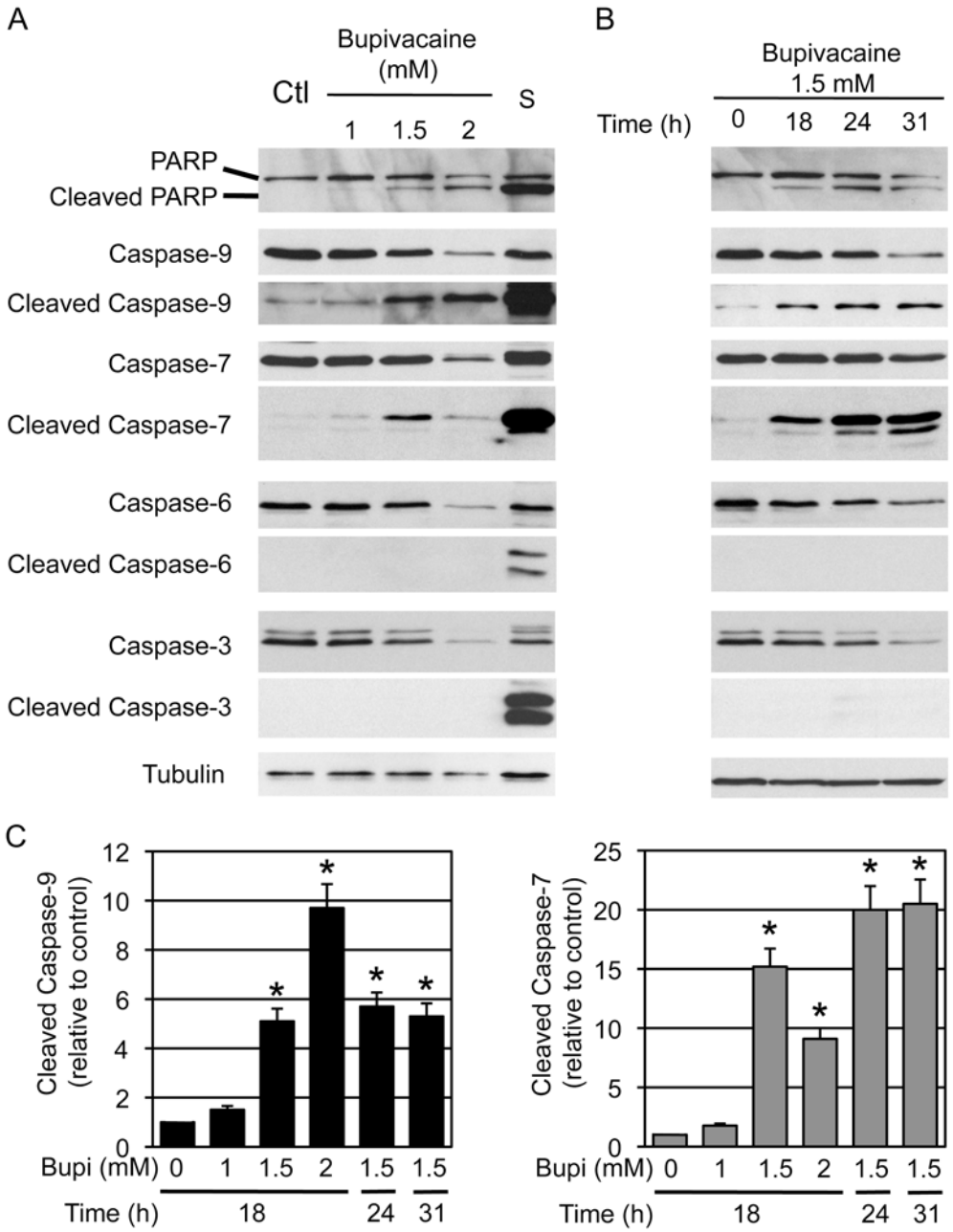
(without bupivacaine and NAC); $P < 0.01$ with one-way analysis of variance of NAC effect with 1 and 1.5mM bupivacaine and $^{\#}P < 0.05$ *with post hoc* unpaired *t*-test *versus* without N-acetyl cysteine (baseline value) for the same bupivacaine concentration, with Holm's procedure. For cl-CSP9 and 7 expression analysis, for 1.5 mM bupivacaine, $*P < 0.01$ with unpaired *t*-test *versus* control (without bupivacaine and NAC); $P < 0.01$ with one-way analysis of variance of NAC effect with 1.5 mM bupivacaine and $^{\#}P < 0.05$ *with post hoc* unpaired *t*-test *versus* without N-acetyl cysteine (baseline value) for the same bupivacaine concentration, with Holm's procedure. (C) Human myotubes were incubated, with 1mM of bupivacaine and without or with the indicated concentrations of NAC. After 18 hours, myotubes were fixed and incubated with anti-CHOP antibody, followed by fluorescently-labeled secondary antibodies. DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and F-Actin with rhodamin-conjugated phalloidin, respectively. The percentage of CHOP-positive nuclei was determined in four independent experiments. Values are the mean ± SEM (n=4); $*P < 0.05$ with unpaired *t*-test v*ersus* control (Ctl); $P < 0.05$ with one-way analysis of variance of NAC effect and $^{\#}P < 0.05$ *with post hoc* unpaired *t*-test *versus* without N-acetyl cysteine (baseline value) for the same bupivacaine concentration, with Holm's procedure. CHOP = C/EBP homologous protein, cl-CSP9 = Cleaved Caspase-9, cl-CSP7 = Cleaved Caspase-7.

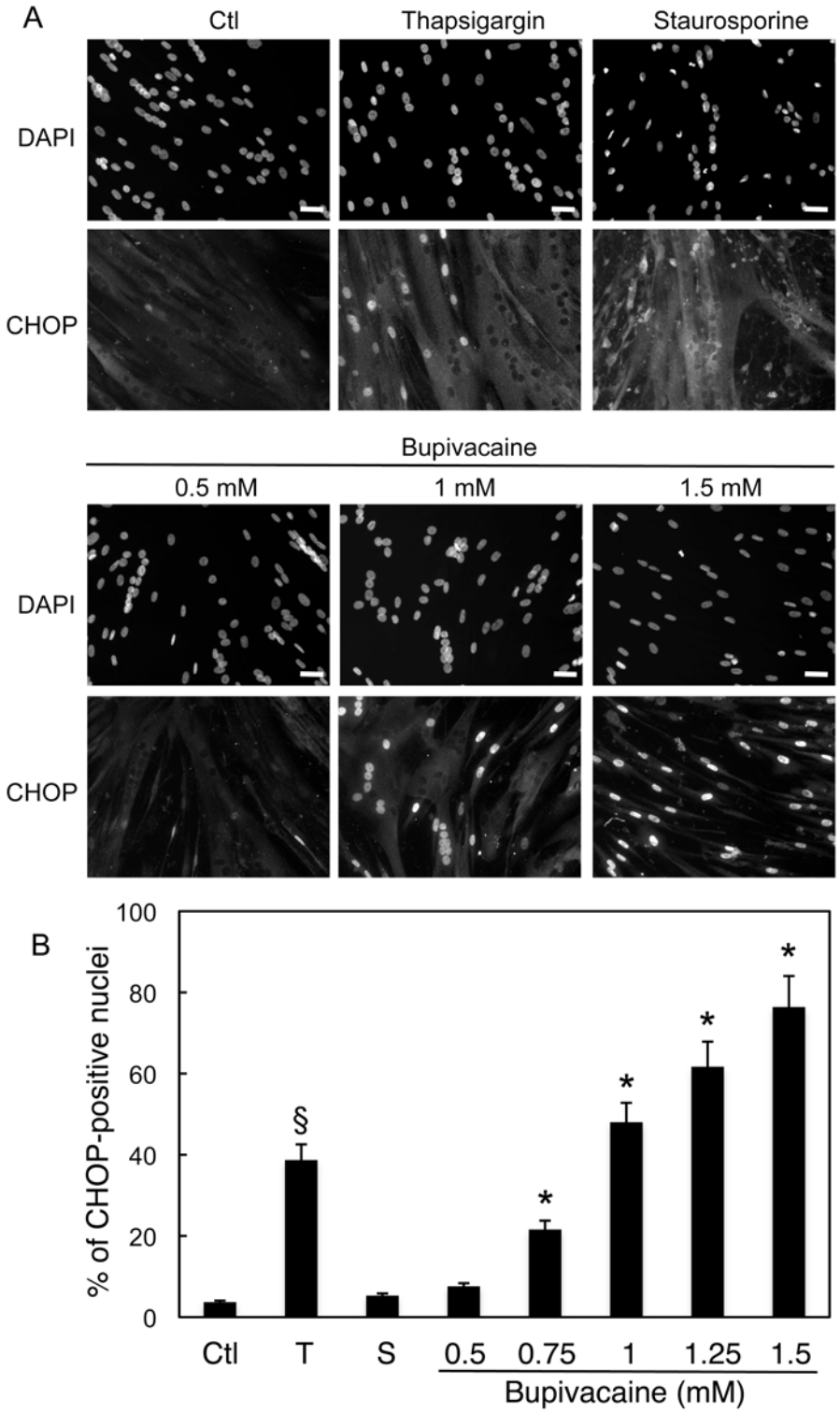
Figure 7: Potentiel mechanism of bupivacaine-induced myotoxicity and N-acetyl cysteine (NAC) cytoprotection in skeletal muscle.

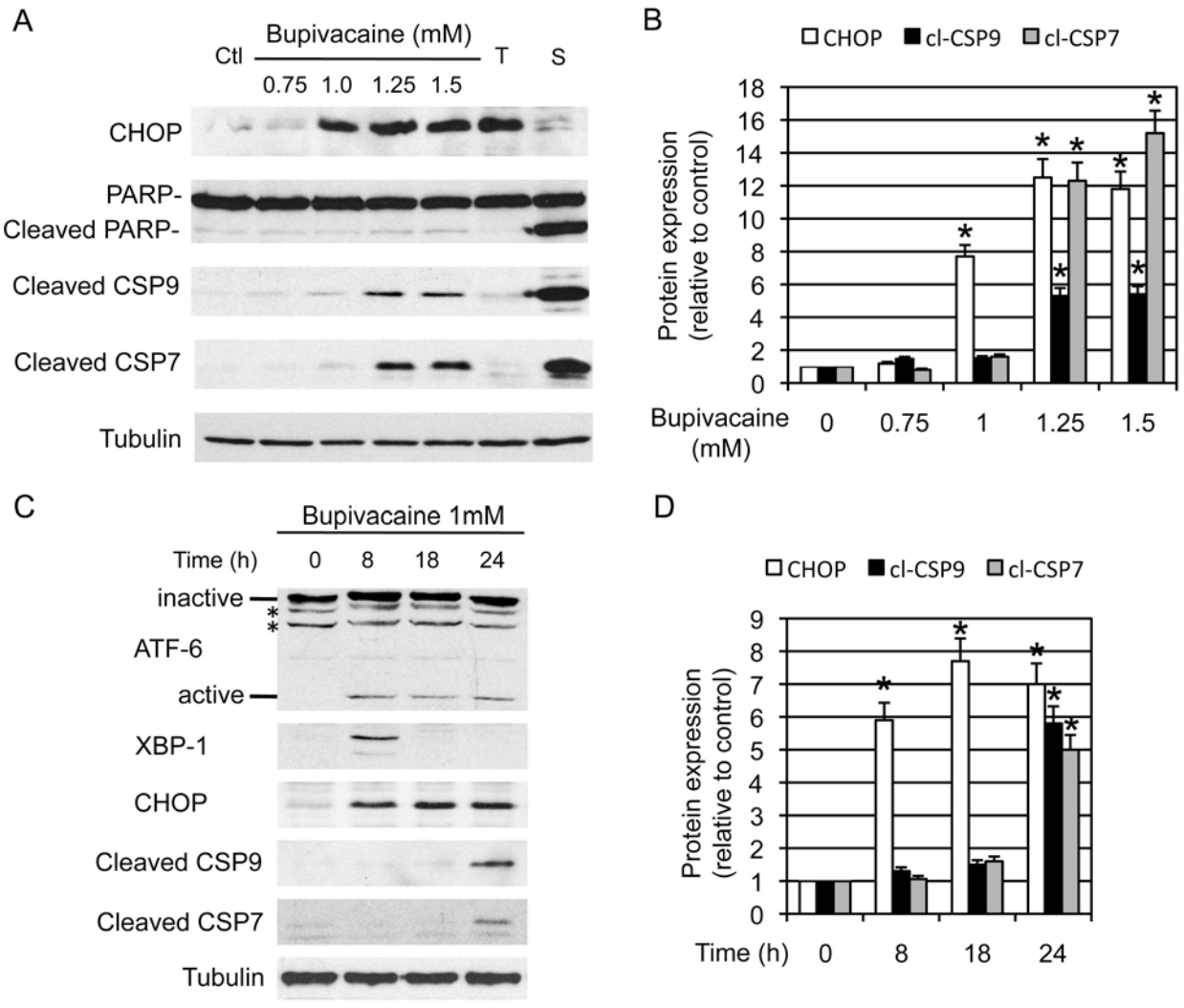
Bupivacaine increases the production of reactive oxygen species (ROS) which induce sarcoplasmic reticulum (SR) stress and the unfolded protein response (UPR) and may cause SR calcium depletion. Bupivacaine sequentially induces ROS production and SR stress leading to apoptosis mediated by the mitochondrial death pathway. Blocking ROS production, the anti-oxidant NAC protects against bupivacaine-induced myotoxicity. CHOP = C/EBP homologous protein, ATF-6 = activating

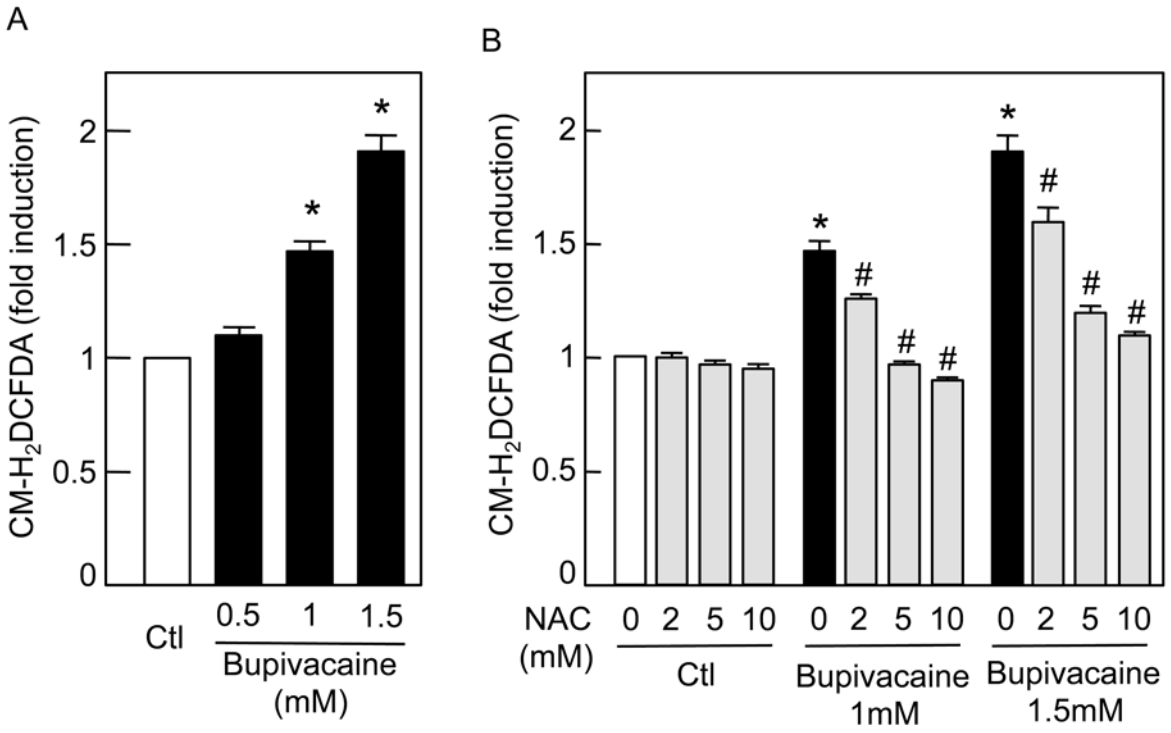
transcription factor 6, XBP-1 = X-box binding protein 1, RyR = channel- ryanodine receptors, SERCA = sarcoplasmic reticulum Ca^{2+} ATPase.

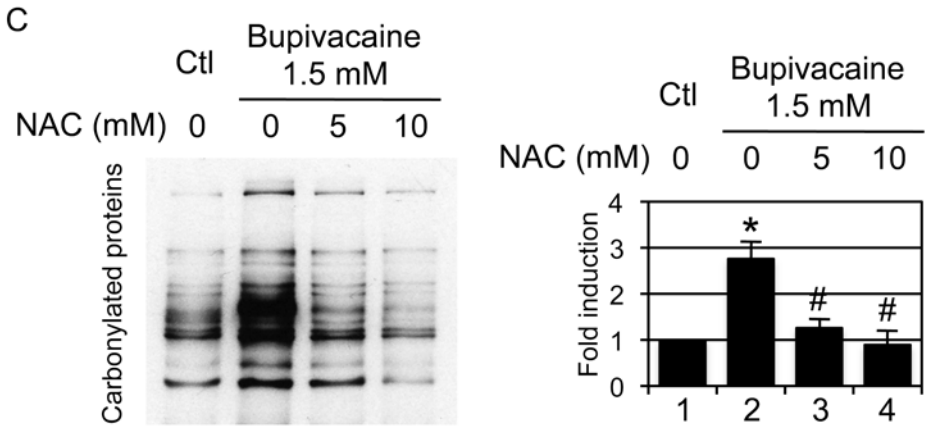

A
Ctl
Bupivacaine (mM)
0.75
1
1.25
1.5
S
actin
DAPI
Cleaved PARP
B
% of apoptotic nuclei
100
80
60
40
20
0
*
*
*
Ctl
18 h
24 h
31 h
Bupivacaine 1.25 mM

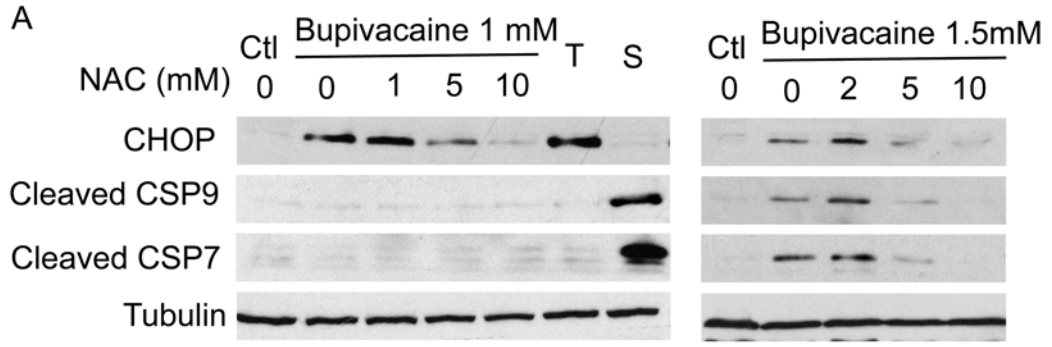

A
Bupivacaine (mM)
Ctl
1
1.5
2
S
PARP
Cleaved PARP
Caspase-9
Cleaved Caspase-9
Caspase-7
Cleaved Caspase-7
Caspase-6
Cleaved Caspase-6
Caspase-3
Cleaved Caspase-3
Tubulin
B
Bupivacaine 1.5 mM
Time (h)
0
18
24
31
C
Cleaved Caspase-9 (relative to control)
Bupi (mM) 0 1 1.5 2 1.5 1.5
Time (h) 18 24 31
Cleaved Caspase-7 (relative to control)
Bupi (mM) 0 1 1.5 2 1.5 1.5
Time (h) 18 24 31

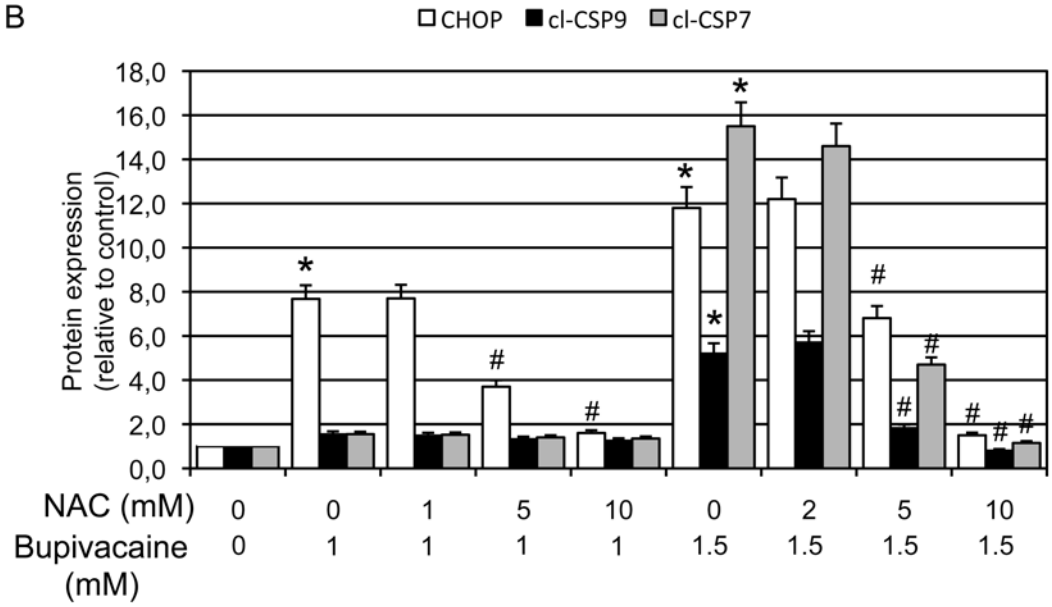

A
Ctl
Thapsigargin
Staurosporine
DAPI
CHOP
Bupivacaine
0.5 mM
1 mM
1.5 mM
DAPI
CHOP
B
% of CHOP-positive nuclei
100
80
60
40
20
0
§
*
*
*
*
Ctl
T
S
0.5
0.75
1
1.25
1.5
Bupivacaine (mM)


A
Bupivacaine (mM)
Ctl
0.75 1.0 1.25 1.5
T
S
CHOP
PARP-
Cleaved PARP-
Cleaved CSP9
Cleaved CSP7
Tubulin
B
CHOP
cl-CSP9
cl-CSP7
Protein expression (relative to control)
Bupivacaine (mM)
0 0.75 1 1.25 1.5
C
Bupivacaine 1mM
Time (h)
0 8 18 24
inactive
ATF-6
active
XBP-1
CHOP
Cleaved CSP9
Cleaved CSP7
Tubulin
D
CHOP
cl-CSP9
cl-CSP7
Protein expression (relative to control)
Time (h)
0 8 18 24


A
CM-H2DCFDA (fold induction)
0
0.5
1
1.5
2
*
*
Ctl
0.5
1
1.5
Bupivacaine
(mM)
B
CM-H2DCFDA (fold induction)
0
0.5
1
1.5
2
*
*
#
#
#
#
#
#
NAC
(mM)
0 2 5 10
0 2 5 10
0 2 5 10
Ctl
Bupivacaine
1mM
Bupivacaine
1.5mM


C
Ctl
Bupivacaine
1.5 mM
NAC (mM) 0
0 5 10
Carbonylated proteins
Ctl
Bupivacaine
1.5 mM
NAC (mM) 0
0 5 10
Fold induction
0
1
2
3
4
*
#
#
1
2
3
4


A
Ctl
Bupivacaine 1 mM
T
S
Ctl
Bupivacaine 1.5mM
NAC (mM)
0 0 1 5 10
0 0 2 5 10
CHOP
Cleaved CSP9
Cleaved CSP7
Tubulin


B
CHOP
cl-CSP9
cl-CSP7
Protein expression (relative to control)
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
NAC (mM) 0 0 1 5 10 0 2 5 10
Bupivacaine (mM) 0 1 1 1 1 1.5 1.5 1.5 1.5

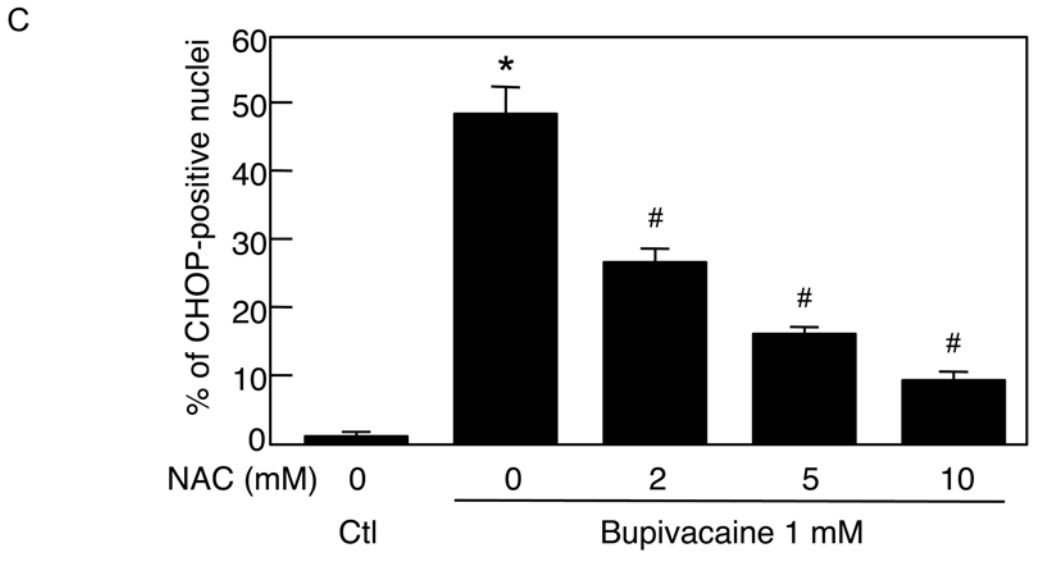
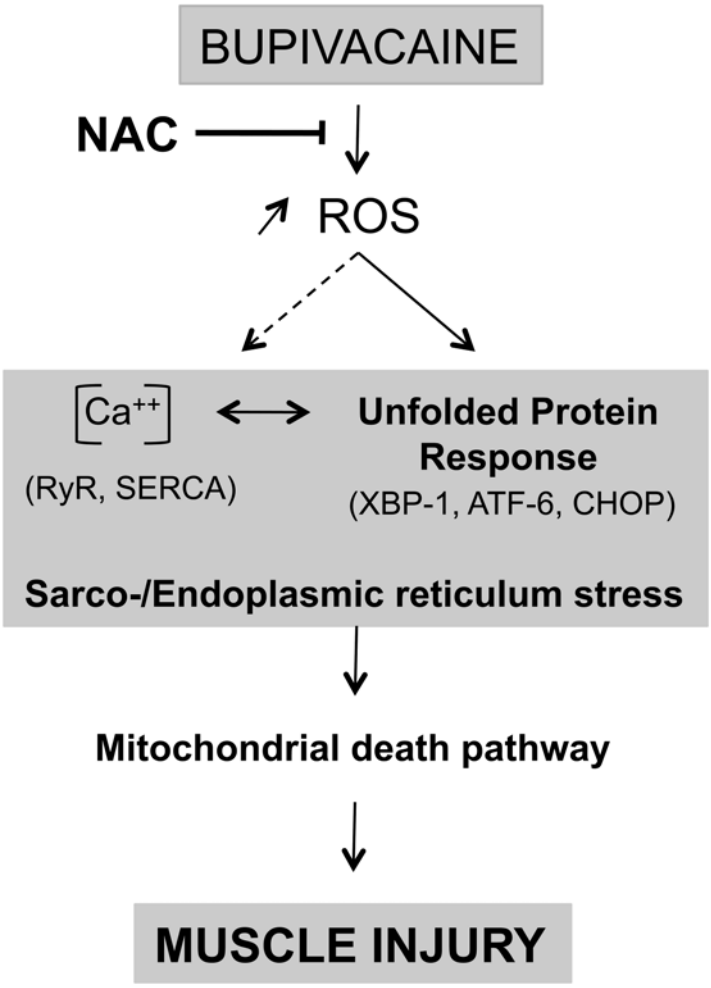

C
% of CHOP-positive nuclei
60
50
40
30
20
10
0
NAC (mM) 0 0 2 5 10
Ctl
Bupivacaine 1 mM

Figure 7


BUPIVACAINE
NAC
ROS
[Ca++]
(RyR, SERCA)
Unfolded Protein Response
(XBP-1, ATF-6, CHOP)
Sarco-/Endoplasmic reticulum stress
Mitochondrial death pathway
MUSCLE INJURY